Figure 1

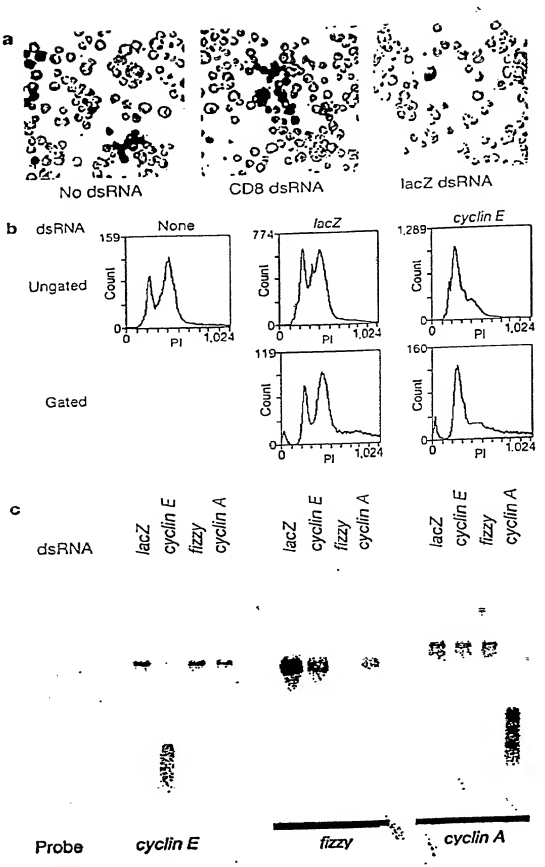

a
No dsRNA
CD8 dsRNA
lacZ dsRNA
b
dsRNA
None
lacZ
cyclin E
Ungated
Gated
Count
PI
159
774
1,289
119
160
0
1,024
c
dsRNA
lacZ
cyclin E
fizzy
cyclin A
Probe
cyclin E
fizzy
cyclin A

Figure 2

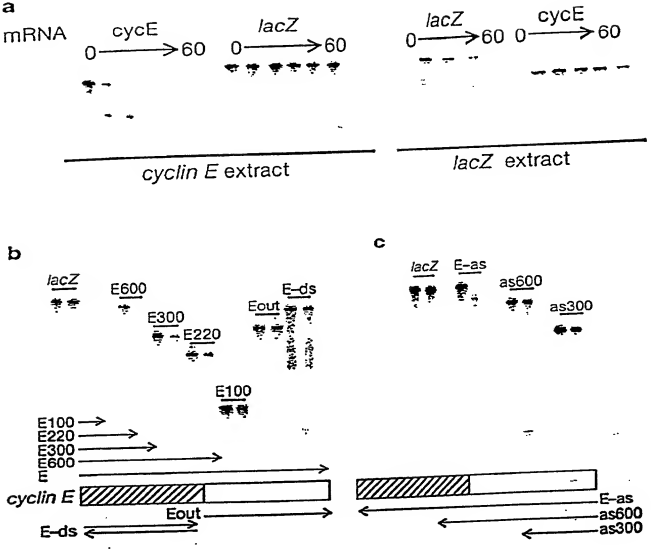

a
mRNA
cycE
0
60
lacZ
0
60
lacZ
0
60
cycE
0
60
cyclin E extract
lacZ extract
b
lacZ
E600
E300
E220
E100
Eout
E-ds
E100
E220
E300
E600
E
cyclin E
Eout
E-ds
c
lacZ
E-as
as600
as300
E-as
as600
as300

Figure 3

Substrate
cyclin E
lacZ
Ca+EGTA
Ca+EGTA+Mn
Ca+Mn
DNAse I
Ca+EGTA
Ca+EGTA+Mn
Ca+Mn
DNAse I

Figure 4

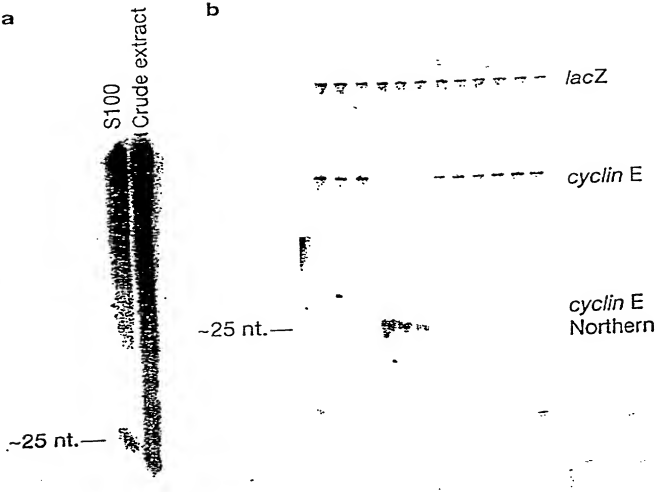

a
S100
Crude extract
~25 nt.—
b
lacZ
cyclin E
cyclin E
Northern
~25 nt.—

Figure 5

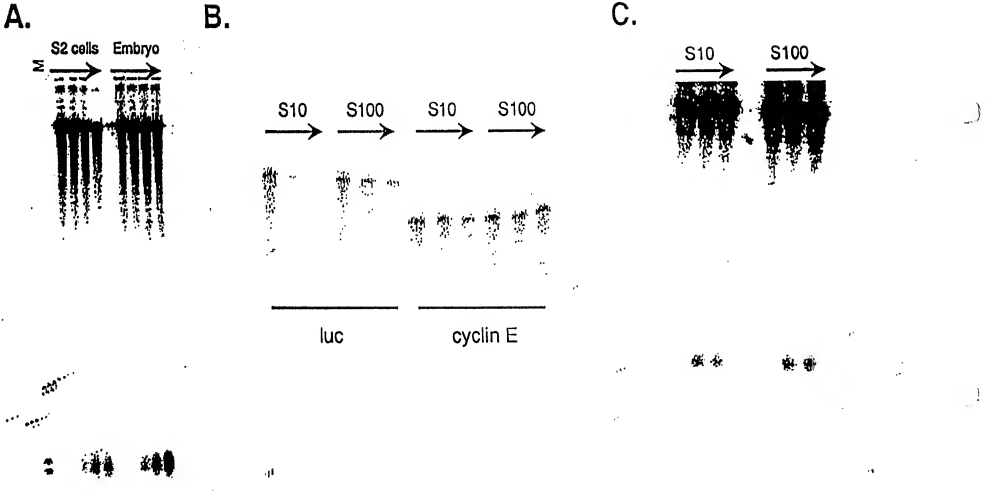


Figure 6a-c

A.
M
S2
Embryo
Drosha
Dicer
Homeless
β-gal
B.
Helicase
ZAP
RIII a
RIII b dsrm
Dicer
Drosha
Homeless
C.
marker
pre-immune
immune
plus peptide
extract

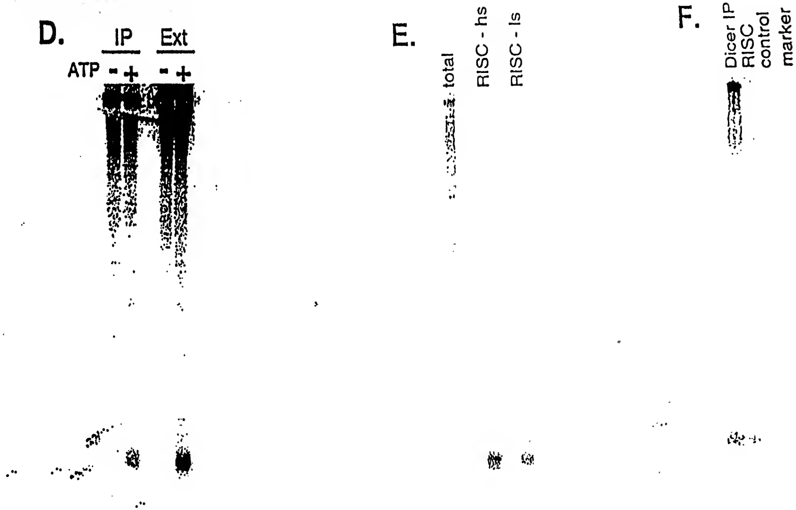

D.
IP
Ext
ATP - + - +
E.
total
RISC - hs
RISC - ls
F.
Dicer IP
RISC
control
marker

Figure 6d-f

Figure 7

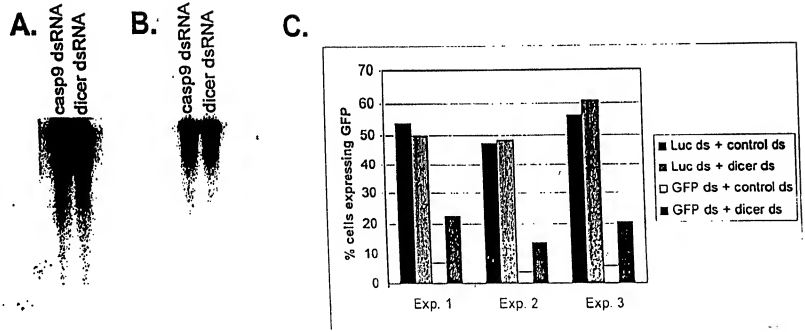

A.
casp9 dsRNA
dicer dsRNA
B.
casp9 dsRNA
dicer dsRNA
C.
% cells expressing GFP
70
60
50
40
30
20
10
0
Exp. 1
Exp. 2
Exp. 3
Luc ds + control ds
Luc ds + dicer ds
GFP ds + control ds
GFP ds + dicer ds

Figure 8A, B

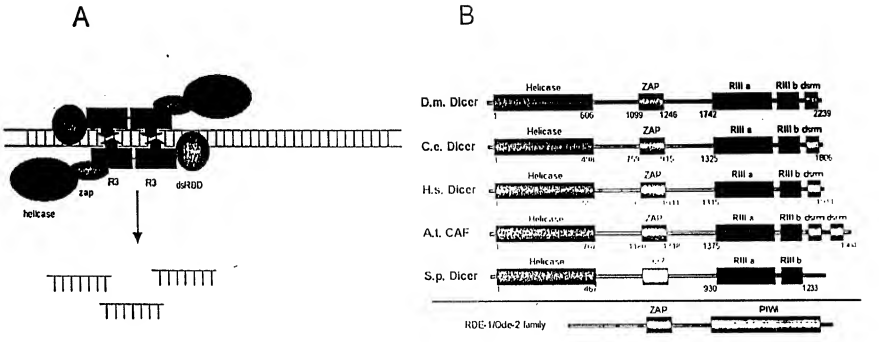

A
zap
R3
R3
dsRBD
helicase
B
D.m. Dicer
Helicase
ZAP
RIII a
RIII b dsrm
1
606
1099
1246
1742
2239
C.e. Dicer
1325
1806
H.s. Dicer
A.t. CAF
S.p. Dicer
930
1233
RDE-1/Ode-2 family
PIWI

Figure 9

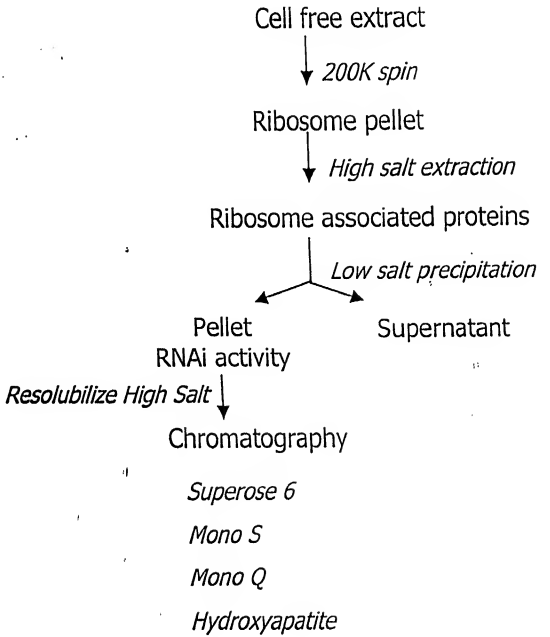

Cell free extract
200K spin
Ribosome pellet
High salt extraction
Ribosome associated proteins
Low salt precipitation
Pellet
Supernatant
RNAi activity
Resolubilize High Salt
Chromatography
Superose 6
Mono S
Mono Q
Hydroxyapatite

Figure 10

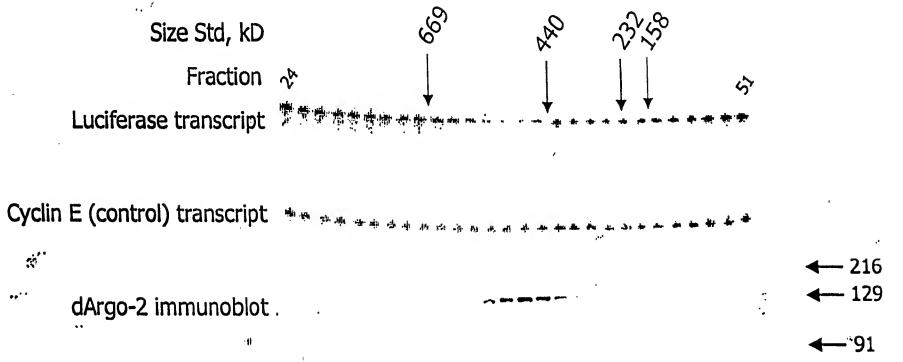

Size Std, kD
669
440
232
158
Fraction
24
51
Luciferase transcript
Cyclin E (control) transcript
dArgo-2 immunoblot
216
129
91

Figure 11

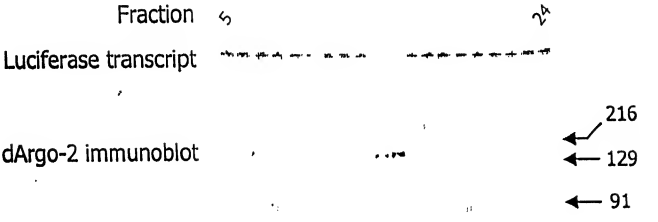

Fraction
5
25
Luciferase transcript
dArgo-2 immunoblot
216
129
91

Figure 12

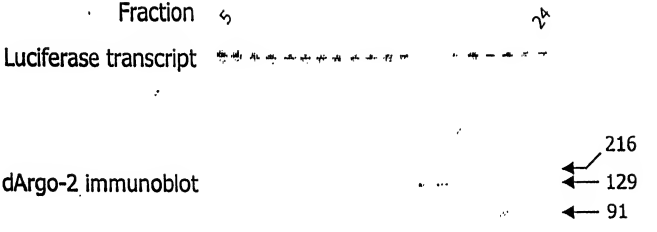

Fraction
5
24
Luciferase transcript
dArgo-2 immunoblot
216
129
91

Figure 13

Figure 14

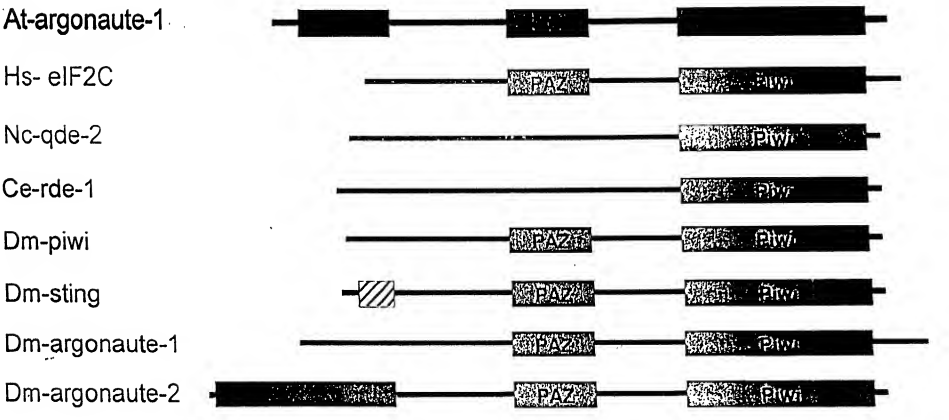

At-argonaute-1
Hs- eIF2C
PAZ
Piwi
Nc-qde-2
Piwi
Ce-rde-1
Piwi
Dm-piwi
PAZ
Piwi
Dm-sting
PAZ
Piwi
Dm-argonaute-1
PAZ
Piwi
Dm-argonaute-2
PAZ
Piwi

Figure 15

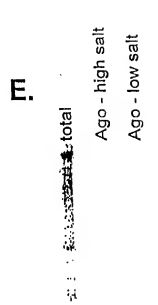


Figure 16

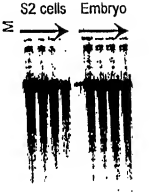
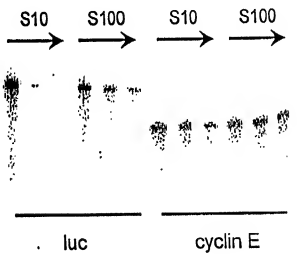


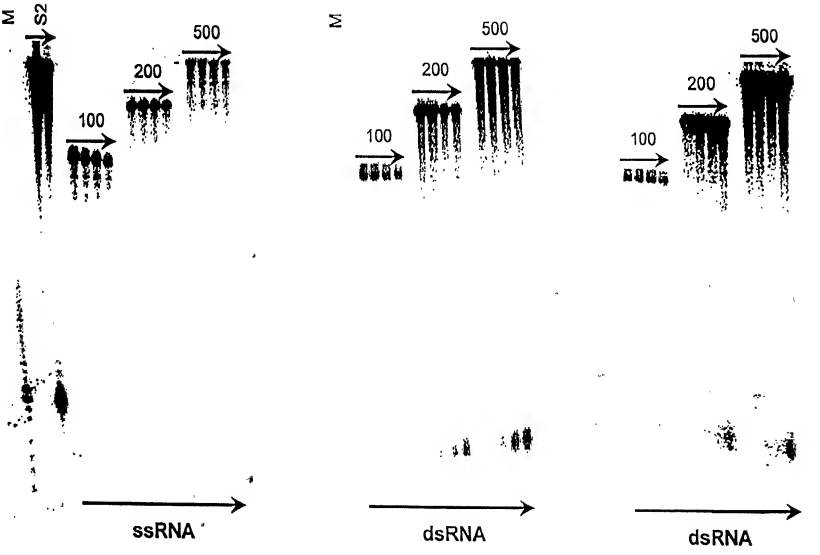
Figure 17



mRNA degradation

dsRNA processing

Figure 18



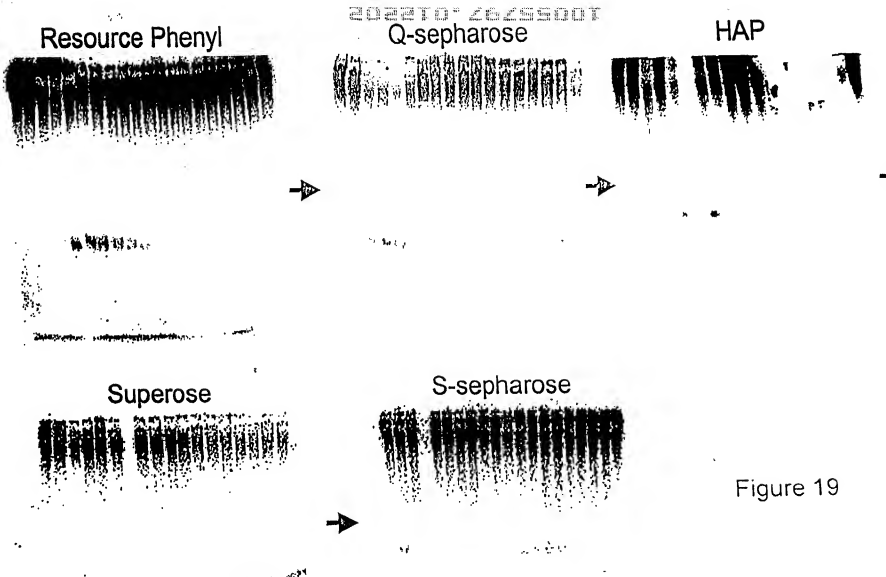


Figure 19

Purification of the 22-mer generating enzyme

Figure 20

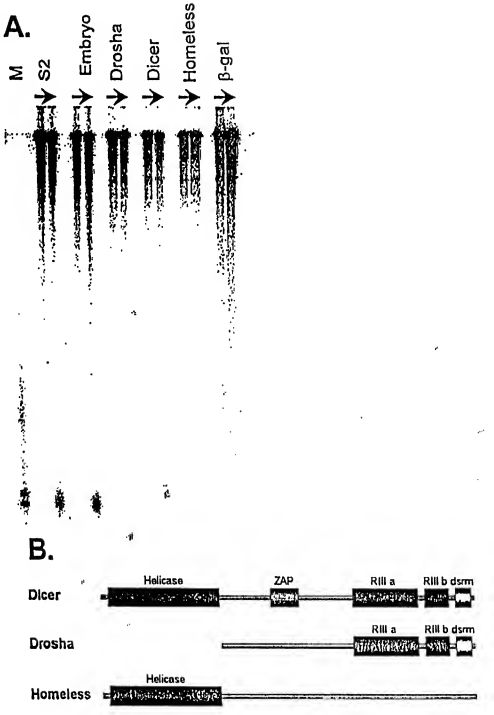

A.
M
S2
Embryo
Drosha
Dicer
Homeless
β-gal
C.
marker
pre-immune
immune
plus peptide
extract
B.
Dicer
Helicase
ZAP
RIII a
RIII b dsrm
Drosha
RIII a
RIII b dsrm
Homeless
Helicase

Figure 21

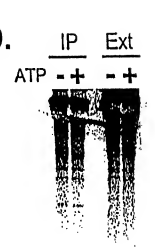


Figure 22

Figure 23

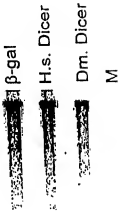
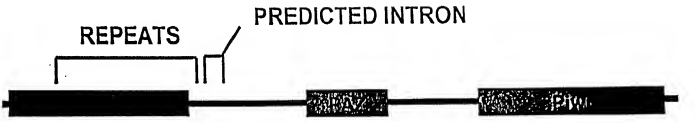


Figure 24

MGKKDKNKKGGQDSAAAPQPQQQQKQQQQRQQQPQQLQQPQQLQQPQQLQQPQQQQQQ
QPHQQQQQSSRQQPSTSSGGSRASGFQQGGQQQKSQDAEGWTAQKKQGKQQVQGWTKQ
GQQGGHQQGRQGQDGGYQQRPPGQQQGGHQQGRQGQEGGYQQRPPGQQQGGHQQGRQG
QEGGYQQRPSGQQQGGHQQGRQGQEGGYQQRPPGQQQGGHQQGRQGQEGGYQQRPSGQ
QQGGHQQGRQGQEGGYQQRPSGQQQGGHQQGRQGQEGGYQQRPSGQQQGGHQQGRQGQ
EGGYQQRPPGQQPNQTQSQGQYQSRGPPQQQQAAPLPLPPQPAGSIKRGTIGKPGQVG
INYLDLDLSKMPSVAYHYDVKIMPERPKKFYRQAFEQFRVDQLGGAVLAYDGKASCYS
VDKLPLNSQNPEVTVTDRNGRTLRYTIEIK**ETGDSTIDLK**SLTTYMNDR**IFDKPMR**AM
QCVEVVLASPCHNKAIRVGR**SFFK**MSDPNNRHELDDGYEALVGLYQAFMLGDRPFLNV
DISHKSFPISMPMIEYLERFSLKAK**INNTTNLDYSR**RFLEPFLRGINVVYTPPQSFQS
APRVYRVNGLSR**APASSETFEHDGK**KVTIASYFHSRNYPLKFPQLHCLNVGSSIKSIL
LPIELCSIEEGQALNRKDGATQVANMIKYAATSTNVRKRKIMNLLQYFQHNLDPTISR
FGIRIANDFIVVSTRVLSPPQVEYHSKRFTMVKNGSWRMDGMK**FLEPKPK**AHKCAVLY
CDPRSGRKMNYTQLNDFGNLIISQGKAVNISLDSDVTYRPFTDDERSLDTIFADLKRS
QHDLAIVIIPQFRISYDTIKQKAELQHGILTQCIKQFTVERKCNNQTIGNILLKINSK
LNGINHKIKDDPRLPMMKNTMYIGADVTHPSPDQREIPSVVGVAASHDPYGASYNMQY
RLQRGALEEIEDMFSITLEHLRVYKEYRNAYPDHIIYYRDGVSDGQFPKIKNEELRCI
KQACDKVGCKPKICCVIVVKRHHTRFFPSGDVTTSNKFNNVDPGTVVDRTIVHPNEMQ
FFMVSHQAIQGTAKPTRYNVIENTGNLDIDLLQQLTYNLCHMFPRCNRSVSYPAPAYL
AHLVAARGR**VYLTGTNR**FLDLKKEYAKRTIVPEFMKKNPMYFV

Figure 25



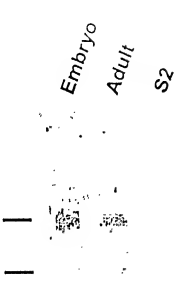


Figure 26

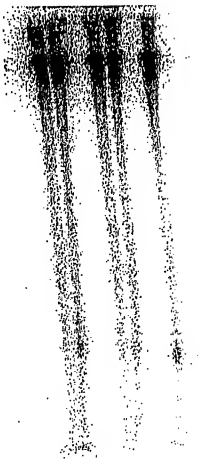


Figure 27

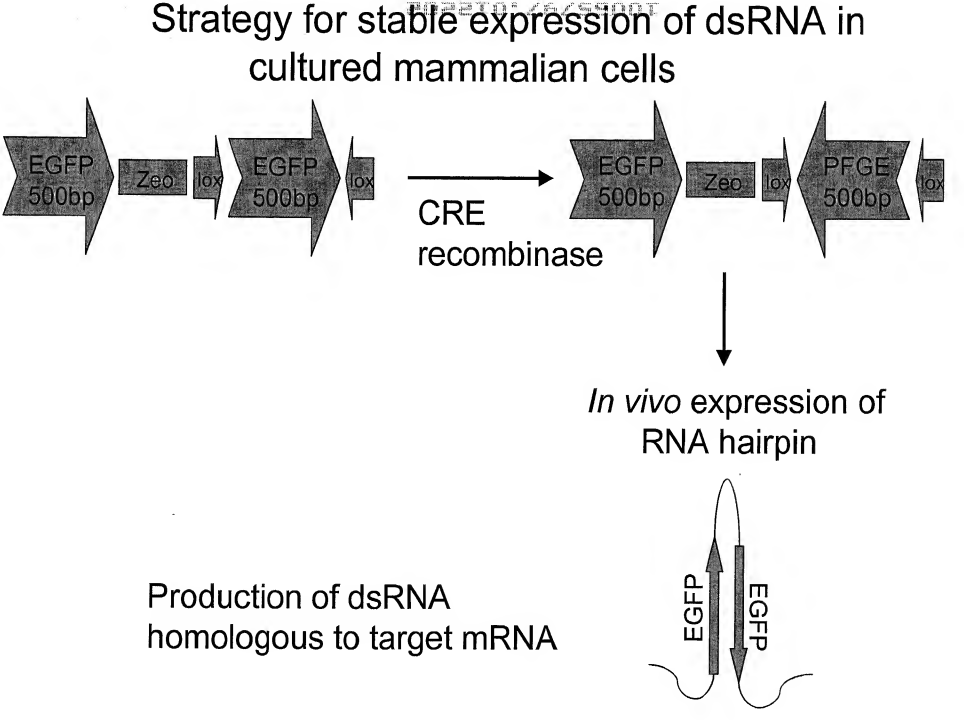
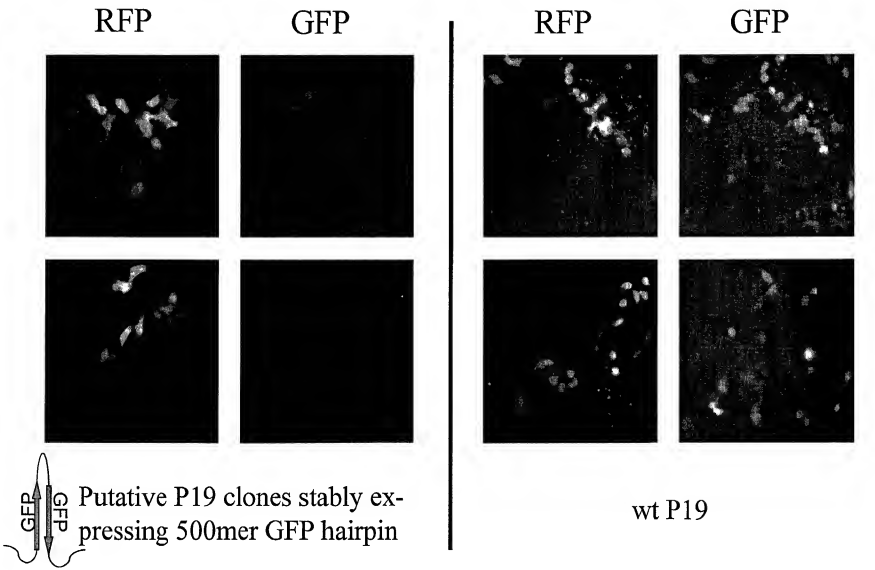

Strategy for stable expression of dsRNA in cultured mammalian cells
EGFP 500bp
Zeo
lox
EGFP 500bp
lox
CRE recombinase
EGFP 500bp
Zeo
lox
PFGE 500bp
lox
In vivo expression of RNA hairpin
Production of dsRNA homologous to target mRNA
EGFP
EGFP

Figure 28

Stable suppression of transgene expression in mammalian cells



Co-transfection with pRFP and pGFP, 42 hrs post-transfection

Figure 29

Dual luciferase assay 21 hrs post-transfection (.4ug dsRNA)

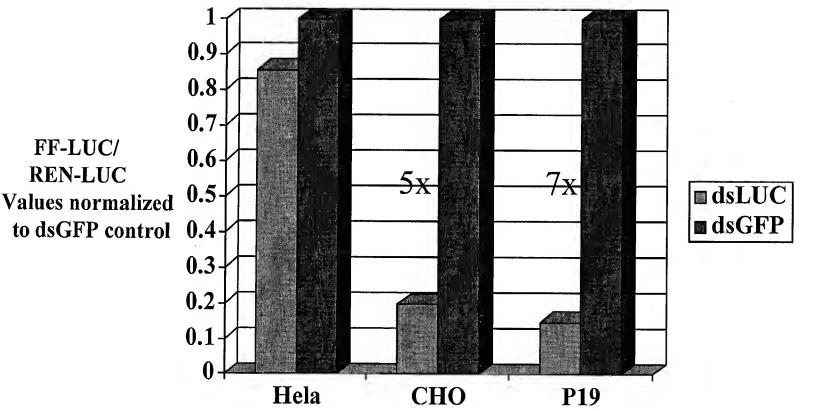


Figure 30

RNAi in ES cells

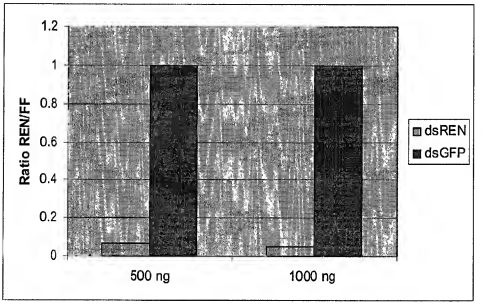
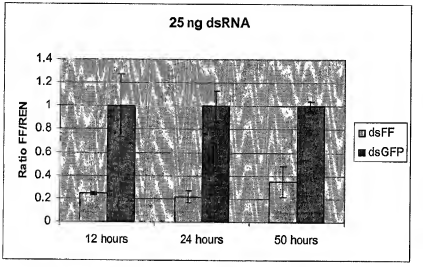


Figure 31

RNAi in mouse embryonic cells (P19)



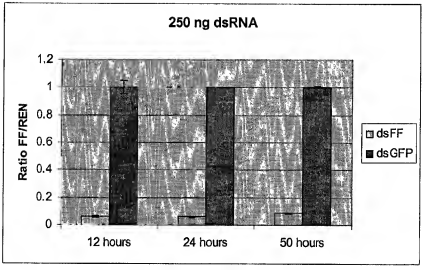


Figure 32

RNAi is post-transcriptional

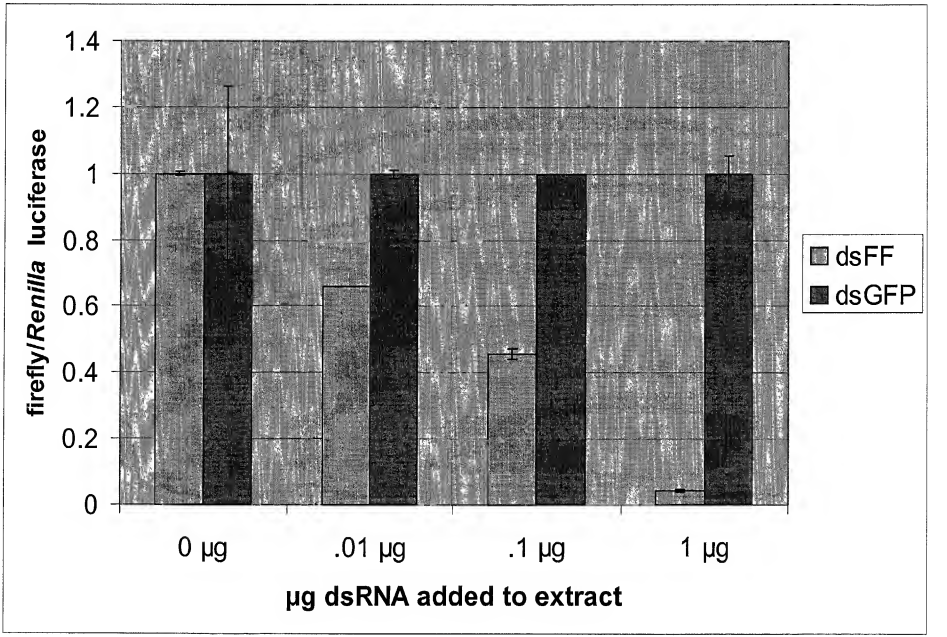
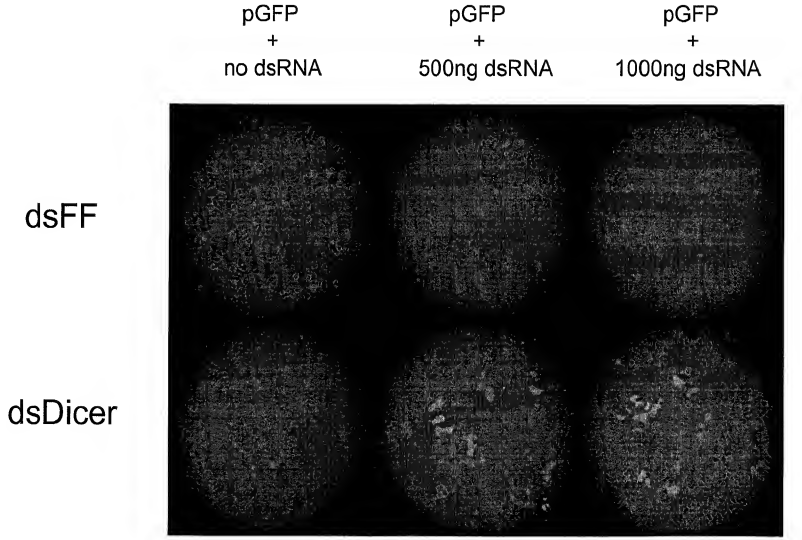


Figure 33



P19 GFP hairpin clone number #10
48hrs post-transfection
Fluorescent microscopy superimposed with bright field

Figure 34

Silencing is specific and requires dsRNA

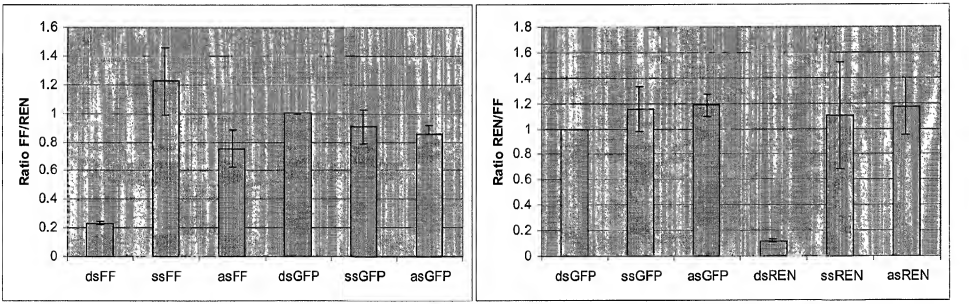


Figure 35

P19 cells soaked with in dsRNA for
12 hrs in 2mL growth medium (alpha MEM, 10% FBS)

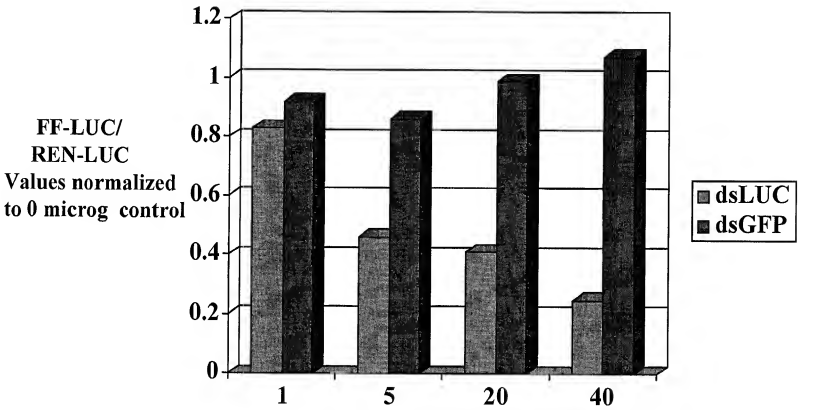
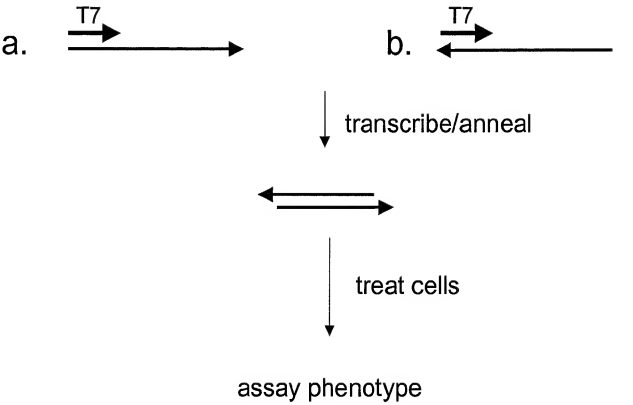


Figure 36

In vitro synthesis of siRNAs by T7 RNA polymerase



DNA synthesis/RNA transcription
~ $16/siRNA versus ~$400/siRNA for chemical synthesis
Brings large-scale projects within reasonable budget range

Figure 37

Luciferase siRNA

Luciferase Let-7 like

Luciferase simple hairpin

Figure 38

Short Hairpin RNAs in Drosophila S2 cells

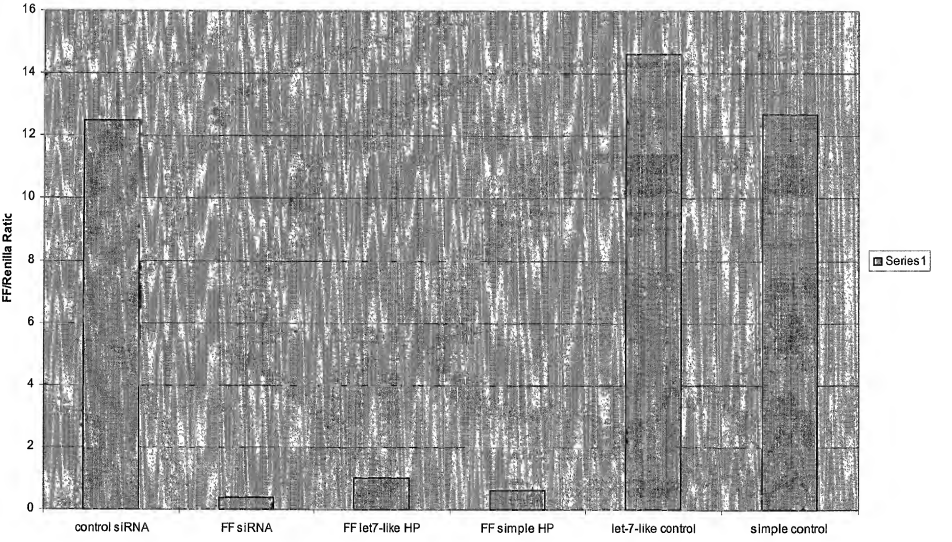


Figure 39

Short Hairpin RNAs in Human 293T cells

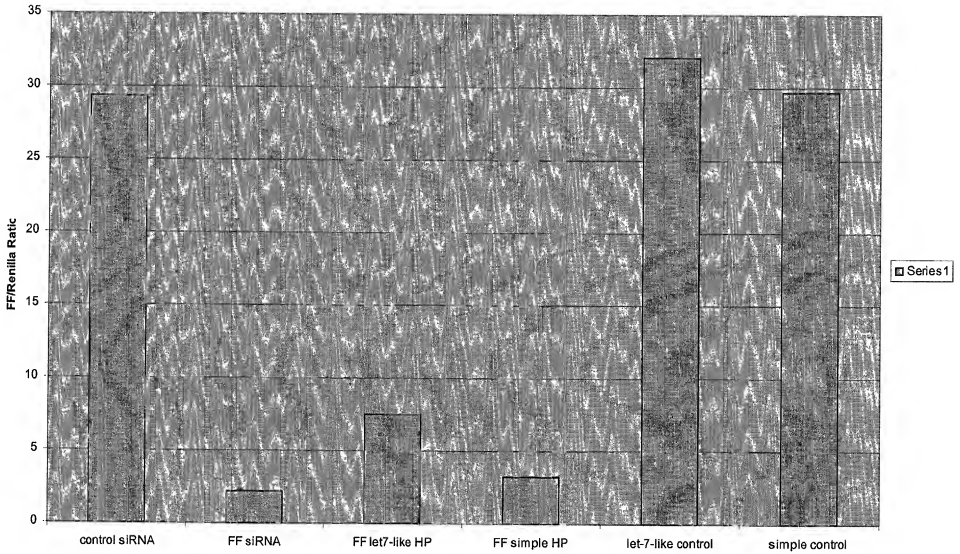


Figure 40

Short Hairpin RNAs in Human HeLa cells

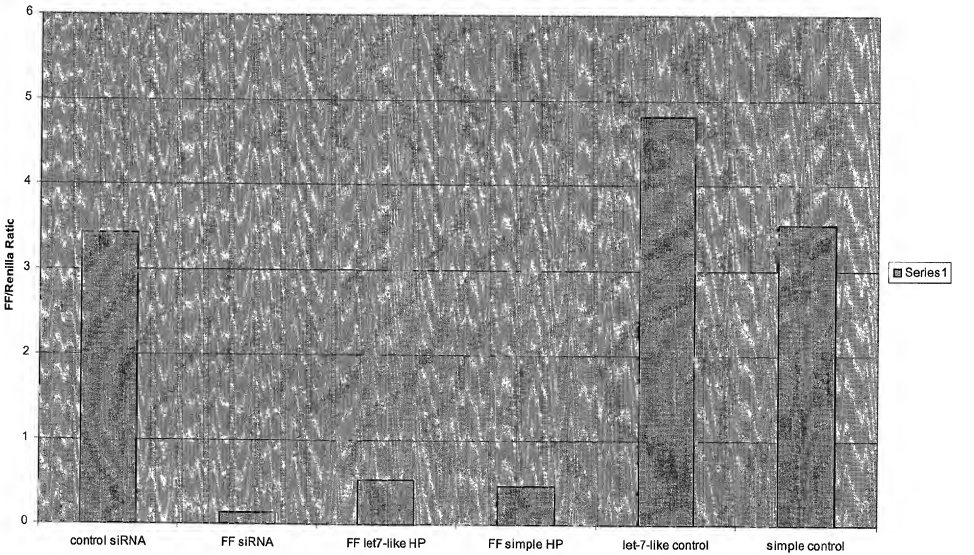


Figure 41

Simultaneous introduction of multiple hairpins does not produce synergy

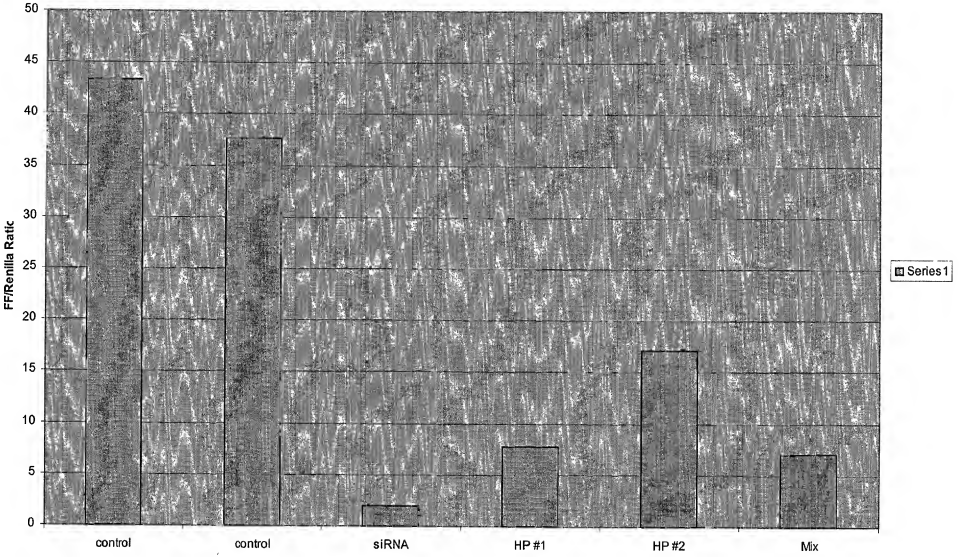
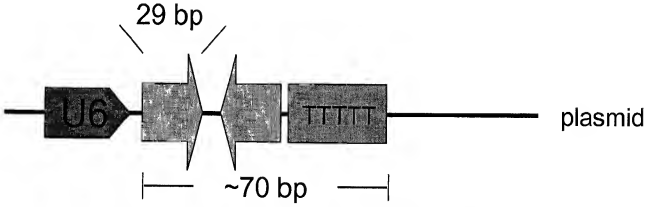


Figure 42

Encoded short hairpins function *in vivo*



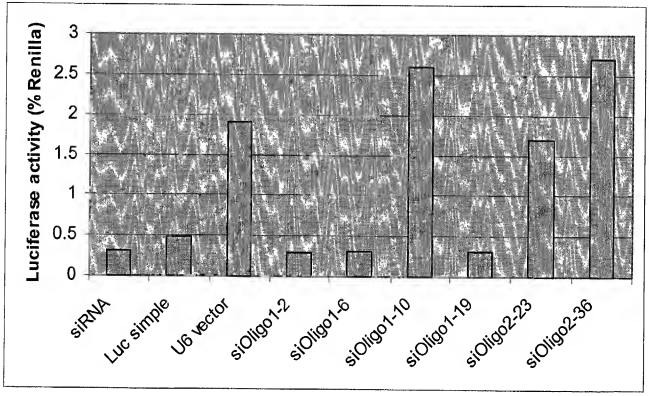


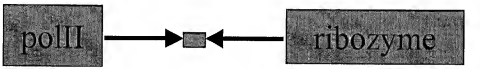
Figure 43

Stable Suppression by short dsRNAs – stable expression strategies

T7 gives site-specific initiation. 3' end formation
Achieved with ribozyme (e.g. hepatitis delta virus ribozyme).

polIII gives site-specific initiation.
Example promoters – U6 snRNA, H1 RNA, SRP RNAs (7SL)
3' end formation
Achieved with native terminator (e.g. TTTTT). Leaves the last TT, so that could be used to pair to transcript.

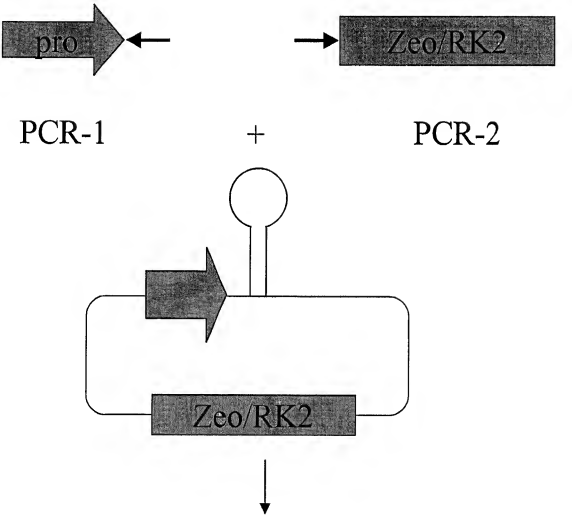
Could also use VA1, tRNA etc but would have to couple with Ribozyme since those promoters need also internal elements.



polII gives site-specific initiation. Example promoters
Would be U1 snRNA promoters, CMV etc…
3' end formationachieved with ribozyme
(e.g. hepatitis delta virus ribozyme).

Figure 44

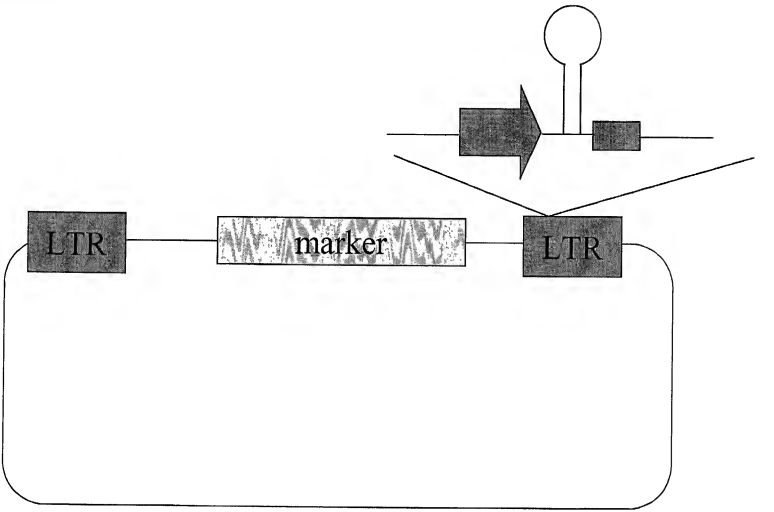
Stable Suppression by short dsRNAs – cloning strategy



Automatic subcloning into vector of choice

Figure 45

MaRX-R



Stable suppression by expressed RNAi

Figure 46

Early Passage PKR -/- MEFs: dual luciferase assay with long dsRNA (~500nt)

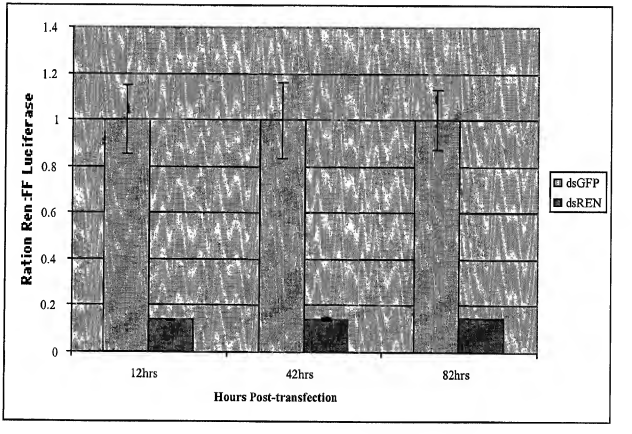


Figure 47

Mouse Tyrosinase Promoter